Introduction

Apolipoprotein (apo) AI, the major HDL protein, plays an important role in reverse cholesterol transport [1], one of the mechanisms for anti-atherosclerosis. A part of apoAI is known to be oxidized by myeloperoxidase (MPO), which is derived from macrophages and neutrophils residing in atherosclerotic lesions [2]. These cells are observed more frequently and at higher density in the lesions of patients with acute myocardial infarction (AMI) as compared to those in patients with stable coronary disease [3]. MPO induces chlorination and nitration of tyrosine residues and/or sulfoxidation of methionine residues in apoAI within atherosclerotic lesions [4,5]. In addition, the apoAI-apoAII heterodimer is produced through a tyrosine-tyrosine bond that is induced through MPO-mediated oxidation [6].

Although plasma MPO activity is reported to be a biomarker of risk for acute coronary syndromes [7], it is not necessarily specific to cardiac disease, since macrophages and neutrophils are activated by a number of infectious and inflammatory disease processes [8]. By contrast, MPO oxidation products, including chlorinated, nitrated and sulfoxidated apoAI, have attracted a great deal of attention as products of MPO catalysis that demonstrate reduced cholesterol acceptor activity upon MPO modification [9]. Thus, an analysis of apoAI oxidation products should yield complementary information to that of MPO activity, and will likely
provide data that are more specific to cardiac disease. Although there is some controversy over whether the apoAI oxidation products induced by MPO impair or enhance reverse cholesterol transport [10,11], they may be useful as biomarkers reflecting plaque instability.

Several analytical methods have been reported for the determination of chlorinated, nitrated, and sulfoxidated apoAI. However, the majority involve isolation of HDL followed by analysis by HPLC [12], mass spectrometry [13,14], or immunoblotting [15].

Here, we characterized the apoAI-apoAII heterodimer, and developed a sandwich ELISA method for its quantification. This study serves as a source of preliminary data for exploring the potential clinical applications of using this assay as part of a panel of cardiac disease biomarkers.

Materials and Methods

Chemicals and blood samples. Unless otherwise stated, all reagents were purchased from Wako Pure Chemical (Tokyo, Japan). Blood samples were drawn with informed consent from apparently healthy volunteers and patients diagnosed with acute myocardial infarction (AMI). The study was approved by the ethics committee of Tokyo Medical and Dental University.

Antibodies. Goat anti-human apoAI and apoAII polyclonal antibodies were purchased from Academy Bio-Medical (Houston, TX). Rabbit anti-human MPO was purchased from Biodesign International (Saco, ME). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG polyclonal antibody was purchased from Medical and Biological Laboratories (Nagoya, Japan). Biotinylated mouse anti-human apoAI monoclonal antibody was purchased from MABTECH (Cincinnati, OH). HRP-conjugated streptavidin and streptavidin-peroxidase were purchased from Sigma Aldrich Japan (Tokyo, Japan), while streptavidin-polyHRP80 was purchased from Stereospecific Detection Technologies (Baesweiler, Germany).

Isolation of HDL. HDL (density 1.063–1.21 g/mL) was isolated from pooled human serum by ultracentrifugation as described previously [16], and dialyzed against 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA-2Na. HDL was applied to a phosphate-buffered saline (PBS)-equilibrated Sepharose CL-6B column (1.5 x 97 cm) to remove contaminated non-HDL lipoproteins. The purified HDL fraction was then concentrated by ultrafiltration.

Immunoblotting. Immunoblotting was performed as previously described [17]. Briefly, serum and purified HDL fractions were separated by SDS-PAGE using 12.5% polyacrylamide gels, and transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA). The blots were then incubated at
room temperature (RT) for 1 h in 20 mM Tris-buffered saline (TBS, pH 7.6) containing 5% skim milk and 0.05% Tween 20. Blots were washed with TBS containing 0.05% Tween 20 (washing buffer), and incubated for 1 h with anti-apoAI or anti-apoAII antibody in TBS containing 0.5% skim milk. Membranes were rinsed three times with washing buffer, and bands containing apoAI or apoAII were visualized using 3,3’-diaminobenzidine tetrahydrochloride and hydrogen peroxide.

Treatment of HDL with MPO. Ten microliters of HDL (approx. 10 μg protein) was incubated at 37°C for 24 h with 10 μL of 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM H₂O₂, 0.2 mM diethylenetriamine pentaacetic acid (DTPA), 0.4 mM L-tyrosine, and 40 nM MPO. The mixture was then analyzed by immunoblotting or by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) R250 to assess the total apoHDL profile.

Removal of free apoAII from human serum. Anti-apoAI antibody (1 mg) was coupled to 0.4 g of CNBr-activated Sepharose 4B (GE Healthcare Japan, Tokyo) according to the manufacturer’s protocol. A portion of the anti-apoAI antibody–conjugated beads (100 μL) was placed into an eppendorf tube, pelleted by centrifugation, and the supernatant was removed. Then, 100 μL of serum diluted 100-fold with PBS containing 0.3% Tween 20 was added to the tube and incubated for 1 h at RT. The supernatant was removed, and the beads were washed twice with PBS containing 0.1% Tween 20. After washing, the bound fraction was eluted with 100 μL of 50 mM glycine-HCl (pH 2.3), and the eluate (90 μL) was immediately neutralized with 360 μL of 0.1 M Tris-HCl (pH 7.6). This solution was further diluted with an equal volume of a 1:4 solution of 50 mM glycine-HCl (pH 2.3) to 0.1 M Tris-HCl (pH 7.6). This final step resulted in a 1 to 1,000-fold total dilution of serum, and these samples were used to determine the concentrations of apoAI-apoAII heterodimers by ELISA, as described next.

Determining the concentration of apoAI-AII heterodimers in human serum. Polystyrene plates (Polysorp, Nunc, Roskilde, Denmark) were coated with 100 μL/well of anti-apoAII antibody (5 mg/mL) in 0.1 M sodium carbonate buffer (pH 9.6) at 4°C overnight. The plates were washed three times with PBS (pH 7.4) containing 0.1% Tween 20 (PBS-Tw) after each of the following incubation steps. Nonspecific binding was blocked by incubating 300 μL of 2% skim milk in PBS-Tw for 2 h at RT. Free apoAII-removed samples were added to each well (100 μL/well) and incubated for 2 h at RT. A control serum obtained from healthy volunteer, from which apoAII was removed, was diluted from 1,000- to 10,000-fold and used to construct a calibration curve. Then, 100 μL/well of biotinylated mouse anti-human apoAI monoclonal antibody diluted with PBS (1 mg/L) was added and incubated for 2 h at RT. Next, 100 μL/well of

Figure 2. ApoAI-apoAII heterodimer formation is induced by MPO catalysis. HDL treated with (+) or without (−) MPO was subjected to SDS-PAGE followed by immunoblotting for apoAI and apoAII (A) or by staining with Coomassie Brilliant Blue (CBB); (B) Arrows indicate the apoAI-apoAII heterodimer. The molecular masses of the standards are listed to the left.
HRP-conjugated streptavidin (1 mg/L) was added, and the plates were incubated for 2 h at RT. After a final wash step, signal was detected using SureBlue Reserve (Kierkegaard & Perry Laboratories, Gaithersburg, MD) for 20 min. The reaction was stopped by adding 100 µL/well of 0.4 M sulfuric acid. Absorbance at 450 nm was measured by SUNRISE RAINBOW-RC (Wako Pure Chemicals, Tokyo, Japan). All samples were assayed in triplicate. The values obtained for experimental samples were expressed as percentages of the concentration for the control normal serum defined as 100.

**Measurement of MPO concentration in human plasma.** An ELISA platform was used to determine MPO concentrations in human plasma. The assay conditions were the same as described above, except that the reagents were tailored to detect MPO. Accordingly, we used an anti-MPO antibody to capture the analyte, a biotinylated-anti-MPO antibody [18] to detect the analyte, and Streptavidin-PolyHRP80 to develop the signal [19]. Purified MPO (Calbiochem-Merck Millipore, Japan) was used as the calibrator.

**Measurement of apoAI concentration in human serum.** ApoAI concentrations in human serum were measured using an immunoturbidimetric assay according to the manufacturer’s protocol (Sekisui Medical, Tokyo, Japan).

**Results**

**An apoAI-apoAII heterodimer in human serum.** Serum obtained from healthy subjects was analyzed by SDS-PAGE followed by immunoblotting with anti-apoAI and anti-apoAII antibodies (Figure 1). Prominent bands were detected at apparent molecular masses of 28 and 17 kDa, corresponding to the apoAI monomer and apoAII dimer, respectively. Under reducing conditions, the apoAII dimer disappeared and a band was observed at ~8 kDa, corresponding to the monomer form of the protein. Additionally, a band was observed with both antibodies under both reducing and non-reducing conditions at an apparent molecular mass of 37 kDa. This band represented a covalent complex of apoAI and apoAII, possibly joined by a tyrosine-tyrosine bond.

**Treatment of HDL with MPO.** We noted that treatment of HDL with MPO resulted in the appearance of higher molecular weight bands detected by both anti-apoAI and anti-apoAII antibodies on immunoblots run under reducing conditions. Not only was the band representing the apoAI-apoAII heterodimer apparently increased in intensity as compared to the same band in untreated HDL samples, but other apoAI- or apoAII-reactive bands were also observed (e.g., at 50 and 80 kDa for apoAI and at 40, 55 and 105 kDa for apoAII; Figure 2A). With respect to CBB staining, although no differences were observed in the intensities of apoAI and apoAII monomers with or without MPO treatment, the apoAI-apoAII heterodimer (37 kDa) and the apoAI-containing complexes at 50 and 80 kDa were slightly increased in intensity after exposure to MPO (Figure 2B).

**Development of an ELISA specific for the apoAI-apoAII heterodimer.** A calibration curve was constructed using a control serum. For this sample, an arbitrary value of 100 units was assigned. After removal free apoAII with anti-apoAI antibody-conjugated beads, bound fractions, diluted 1,000- to 10,000-fold were tested using the ELISA method.

**Table 1. Increased reactivity between MPO treated apolipoproteins and respected antibodies**

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<thead>
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<th>relative slope</th>
<th>heterodimer</th>
<th>apoAI</th>
<th>apoAII</th>
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<tr>
<td>a. MPO−</td>
<td>0.0028</td>
<td>0.140</td>
<td>0.0042</td>
</tr>
<tr>
<td>b. MPO+</td>
<td>0.0133</td>
<td>0.209</td>
<td>0.0109</td>
</tr>
<tr>
<td>b/a</td>
<td>4.75</td>
<td>1.49</td>
<td>2.60</td>
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From these data, linearly increased absorbance was obtained \((r=0.9997)\). Inter-assay reproducibility for all processes including the removal step was 4.8\% (CV) for an average value of 80.3 (arbitrary units) of the apoAI-apoAII heterodimer in plasma obtained from a normal subject.

Next, additional samples of healthy human serum were serially diluted for a total of six concentrations. Then, those samples were removed free apoAII and the apoAI-apoAII heterodimer was measured by ELISA using the standard control serum to construct the calibration curve (Figure 3). The apoAI-apoAII heterodimer levels of the diluted samples were nearly proportional to the dilution factors, indicating that the present ELISA method, including the removal step, was a useful tool to quantitatively measure the apoAI-apoAII heterodimer in serum samples.

**Reactivity of antibodies with MPO-treated apoAI and apoAII.** To investigate the observed increases in the intensities of immunoreactive bands representing monomeric apoAI and apoAII after MPO treatment of HDL (Figure 2A), MPO-treated and -untreated HDL were diluted to various concentrations with PBS and subjected to ELISA analyses for these analytes (Figure 4). The relative slope of each analyte involved in HDL treated with or without MPO was indicated in Table 1. The data revealed that the concentration of the apoAI-apoAII heterodimer increased \(-4.8\)-fold after MPO treatment. In addition, signals for apoAI and apoAII alone were also increased by 1.5- and 2.6-fold, respectively. Together, these data roughly indicate that the reactivity of the anti-apoAI and anti-apoAII antibodies with their protein targets was enhanced by \(\sqrt{1.5}=1.23\)- and \(\sqrt{2.6}=1.61\)-fold, respectively, after MPO treatment. Therefore, in order to determine the actual increase in apoAI-apoAII heterodimer concentration, we first took into account the enhanced reactivity of the antibodies to apoAI and apoAII. Accordingly, we calculated that the heterodimer concentrations were increased \(-2.4\)-fold after MPO treatment.

**Measuring the apoAI-apoAII heterodimer in human serum.** The relative concentrations of the apoAI-apoAII heterodimer in sera obtained from 8 healthy individuals (1 man and 7 women) ranged from 32 to 110 arbitrary units. A significant correlation between the amounts of apoAI-apoAII heterodimer and apoAII \((r=0.763)\), but not apoAI \((r=0.093)\), was observed in these subjects (Figure 5). Interestingly, the heterodimer levels measured in healthy subjects were modestly but significantly different from those detected in patients urgently hospitalized for acute myocardial infarctions (AMI) (Table 2). However, the apoAI-apoAII heterodimer levels in the latter group were reduced to those seen in the healthy subjects 72 h after treatment for the ischemic event. A significant correlation between the concentrations of the apoAI-apoAII heterodimer and MPO \((n=16, r=0.831)\) was observed (data not shown).

**Discussion**

When freshly-obtained healthy human serum was subjected to immunoblotting analysis using anti-apoAI and anti-apoAII antibodies, a non-reducible band containing both apoAI and apoAII was identified with an apparent molecular mass of 37 kDa, indicating that the apoAI-apoAII heterodimer was present. As determined by semi-quantifiable densitometric analysis of the immunoblotting patterns, the amounts of apoAI and apoAII that were bound...
in the heterodimer represented approximately 2% and 3% of the total apoAI and apoAII, respectively. Considering the normal levels of apoAI and apoAII in serum, (~120‒160 mg/dL and 25‒35 mg/dL, respectively) and the molecular masses of the monomers (28,300 and 8,700, respectively), the data indicate that one molecule each of apoAI and apoAII join together to form the apoAI-apoAII heterodimer.

The intensity of the apoAI-apoAII heterodimer band observed by immunoblotting was clearly increased after treatment of HDL with MPO. This observation was confirmed by SDS-PAGE followed by CBB staining of the gel. In addition, two prominent bands with molecular masses of 50 and 80 kDa were observed to increase in intensity after MPO treatment. However, as these bands were only observed using the anti-apoAI antibody or by CBB staining, we surmised that they represented an apoAI homodimer and homotrimer, respectively. Therefore, MPO treatment induced tyrosine-tyrosine bond formation, not only between apoAI and apoAII, but also between apoAI and apoAI. It remains to be determined whether the tyrosine-tyrosine bond is also induced intramolecularly within apoAI. Similarly, three prominent bands with molecular masses of 40, 55, and 105 kDa increased after MPO treatment. However, these bands were only detectable using the anti-apoAI antibody. Although the composition of these bands was not identified, it is likely that they contained protein complexes, joined by tyrosine-tyrosine bonds, those included apoAII but not apoAI, e.g., an apoE-apoAII heterodimer and apoAII polymer. Finally, immunoblotting also revealed that the bands representing apoAI and apoAII monomers increased in intensity after MPO treatment. In this case, however, a similar increase was not observed by CBB staining. Together, these data suggested the possibility that the apoAI and apoAII monomers nitrate and/or chlorinate by MPO demonstrated improved reactivities with their respective antibodies, as has been previously described [20].

Insufficient sensitivity was obtained when the apoAI-apoAII heterodimer in serum was directly measured by sandwich ELISA, since the abundant free serum apoAII (not form the complex with apoAI) engaged most of the anti-apoAII antibody used to capture the analyte. Therefore, we added a removal step using anti-apoAI antibody-coupled beads that allowed us to obtain a sufficiently sensitive concentration-dependent absorption curve. To evaluate the quantitative capacity of the assay, pre-diluted serum samples were taken through the analysis, including the removal step. The results yielded apoAI-apoAII heterodimer concentrations that were roughly proportional to the sample dilution. Thus, it is likely that the removal step did not adversely impact the precision of the assay.

ApoAI-apoAII heterodimer levels were measured in serum specimens obtained from 8 healthy individuals. The highest heterodimer concentration was about 3-fold greater than the lowest one, suggesting that the heterodimer existed in a wider concentration range as compared to apoAI and apoAII alone. Although there was no correlation between the heterodimer and apoAI concentrations in normal human serum (r=0.093), a relatively high correlation (r=0.763) was observed between the

<table>
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<th>Table 2. Comparison of apoAI-apoAII heterodimer levels between patients with AMI and normal subjects</th>
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<tr>
<td>Normal (n=12)</td>
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<tr>
<td>apoAI-apoAII (arbitrary unit)</td>
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*AMI: acute myocardial infarction  **comparison between Normal and AMI  ***0h: during percutaneous transluminal coronary angioplasty (PTCA); 72h: 72 hours after PTCA (mean±SD)
heterodimer and apoAII concentrations. These data suggest that the formation of apoAI-apoAII heterodimer is induced in the particles of Lp-AI/AII—HDL including apoAI with apoAII—but not Lp-AI—HDL including apoAI without apoAII [21]. Some studies have attempted to use serum MPO activity directly as a biomarker of atherosclerotic disease [7,22,23]. We found that MPO and apoAI-apoAII heterodimer concentrations demonstrated a relatively high correlation both in AMI patients and normal subjects, indicating that the heterodimer could also be useful as a biomarker of atherosclerotic disease. In addition, it is possible that apoAI-apoAII heterodimer formation could be more specific to atherosclerotic lesions as compared with MPO, which is abundantly secreted from neutrophils in a variety of inflammatory settings.

In conclusion, the apoAI-apoAII heterodimer existed in HDL obtained from normal human serum, and its concentration was increased after in vitro

**Figure 4.** HDL incubated with (●) and without (○) MPO was enriched for apoAI using an affinity matrix. Then, the samples were diluted to give the indicated ratio (100%, 50%, and 20% for apoAI-apoAII heterodimer and apoAII; 2% and 1% for apoAI). Concentrations of the apoAII-apoAII heterodimer (a), apoAI (b), and apoAII (c) in the samples were measured by ELISA.

**Figure 5.** Correlation between concentrations of apoAI, apoAII, and the heterodimer in serum. Serum obtained from 8 normal subjects was subjected to ELISA analysis to measure the concentrations of apoAI (A), apoAII (B) and the heterodimer. The Spearman’s correlation coefficient (r) is shown.
oxidation by MPO. Measurements of heterodimer formation may be useful, on par with that of MPO activity, as a biomarker for atherosclerotic disease. The development of a high-throughput assay will be required to confirm this possibility.

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